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14. ABSTRACT Use of the pyridostigmine bromide during the 1991 Gulf War has been implicated as a contributing factor for increased risk of Gulf War Illness (GWI). Stress and genetic factors are postulated to modulate the effects of PB. We have proposed to use mRNA-seq to study the response to PB exposure and modulating factors, using zebrafish as a model organism. In the past year, we have studied the dose-response to PB in larval and adult zebrafish at the biochemical and transcriptomic levels, validated a methods of treatment for inducing stress in adult zebrafish, and conducted a preliminary study to identify unique effects of PB by comparison with the effects of other acetylcholinesterase inhibitors. Our preliminary results have identified transcripts altered due to the effects of PB or genetic background, with the effects of genetic background being the larger effect of the two. Sequencing of additional samples is currently underway and data analysis will be ongoing. We propose a time course study and investigation of PB and stress treatments in adult fish as additional work that will help elucidate the effects of PB that may be related to the symptoms of GWI. We also propose to perform additional experiments, with existing funds, to begin examining more toxins for their effects on genomic measurements in zebrafish.					
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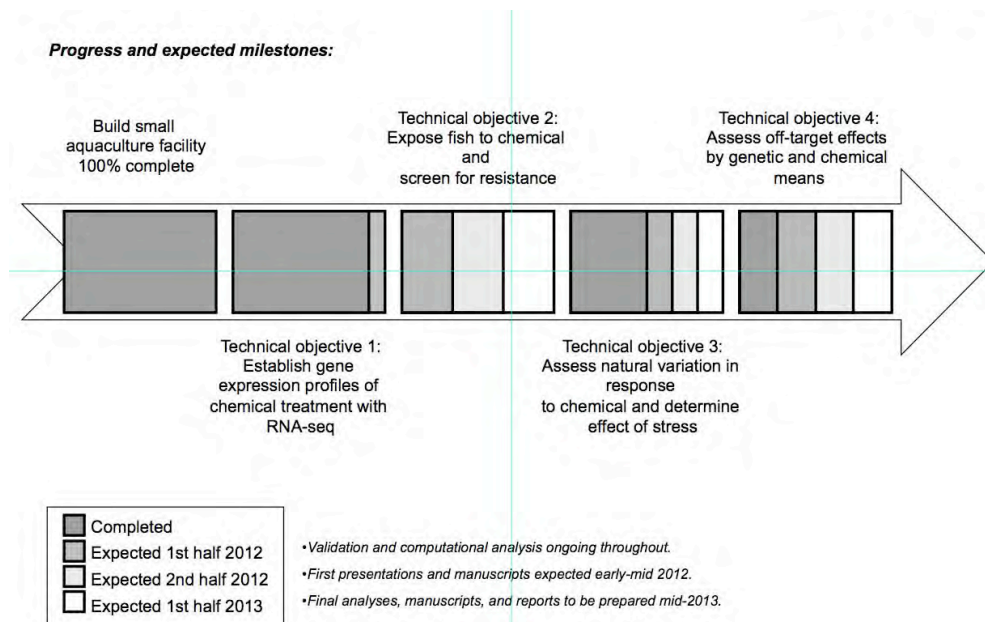
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EXECUTIVE SUMMARY

Treatment of soldiers of the 1991 Gulf War with the drug pyridostigmine bromide for pretreatment against nerve agents has since been associated with increased incidence of Gulf War Illness. The primary objectives of our research are to use zebrafish as a model organism for the characterization of the effects of pyridostigmine bromide (PB) on gene expression using unbiased, high-throughput techniques, specifically mRNA-seq. Additionally, we aim to study the PB response in the presence of factors that may modulate the effects of PB as indicated by epidemiological and experimental evidence, such as genetic variation or stress. Over the course of the past year, we have made great strides towards achieving these goals by conducting PB dose-response experiments in larval and adult zebrafish of multiple strains (representing natural variation), validating a protocol for inducing stressful conditions in adult zebrafish, and initiating a study of the unique effects of PB by comparison with effects from treatment with other acetylcholinesterase inhibitors. We have requested a no-cost extension for the next 24 months (9/21/11 - 9/20/13) to extend these studies substantially further than we originally proposed, as the technologies for genomic measurements have improved immensely. This will allow a much larger amount of data (e.g., deeper RNA sequencing on more samples and an increase in the number and types of toxins that we can screen, as well as the measurement of epigenetic changes) within our existing funds. During the next year, we plan to expand upon the foundation established by this work by investigating the long-term effects of PB following transient exposures to PB in the presence or absence of stress. We also intend to use new methodologies to validate our results, phenotyping of motility and behavioral traits, and studying epigenetic mechanisms of action. By addressing the problem in multiple ways, we hope to uncover novel insights into the mechanism by which PB exposure is related to the pathogenesis of Gulf War Illness. As these experiments are done in the next year, we will begin extending them to additional toxins, using the same approaches.

Contract No. W81XWH-09-1-0715 / Proposal #08273008
 HudsonAlpha Institute for Biotechnology
 Richard M. Myers, PI



INTRODUCTION

Pyridostigmine bromide (PB) was given to approximately 250,000 soldiers of the 1991 Gulf War as a prophylactic against potential nerve agent attack (1). Pyridostigmine is a quaternary ammonium compound that reversibly binds acetylcholinesterase, thereby protecting the enzyme from being bound by nerve agents, which are irreversible acetylcholinesterase inhibitors. Use of PB has since been positively associated with an increased incidence of Gulf War Illness and increased severity of the symptoms of Gulf War Illness, such as fatigue, musculoskeletal pain, mood and cognitive disorders, dermatological irritation and gastrointestinal effects (2). Recent epidemiological evidence suggests that for soldiers in the most forward deployments, PB exposure was the greatest factor associated with Gulf War Illness (3). Research also suggests that there are potential interactions between the effects PB and additional factors, specifically stress and genetics. Although the precise mechanism is unclear, treatment with PB concurrent with stressful conditions has been shown to result in aberrant behavioral patterns and altered gene expression, enzyme activities and/or reactive oxygen species levels in the brain and muscle (4-6). Genetic variation has also been associated with poor health in Gulf War veterans. For example, variants of paraoxonase 1 and butyrylcholinesterase that result in slower detoxification of organophosphates and PB have been found to be associated with an increased likelihood of Gulf War Illness symptoms in veterans (7, 8). Therefore, it is our objective to study in an unbiased manner the dose-dependent effects of PB, as well as to investigate the potential influences of stress and genetics, using zebrafish as a model organism. Furthermore, we will expand this approach to study additional toxins towards the second half of the remaining funding period.

BODY

The goals for our project are to elucidate the effects of pyridostigmine bromide using genetic and genomic tools (W81XWH-09-1-0715) to generate transcriptional profiles that further our understanding of the mechanistic basis of the relationship between PB treatment and Gulf War Illness, as well as address the effects of stress and genetic variation. Over the duration of the past year, our specific aims have been to continue to grow of our aquaculture facility and zebrafish populations; to determine the appropriate treatment regimens for administration of PB and induction of stress; and characterize the effects of PB on gene expression profiles in larval and adult zebrafish using mRNA-seq as an unbiased, high-throughput method.

We initially proposed to study the effects of PB primarily through transcriptome sequencing. However, due to delays in the construction of the aquaculture facility early in the project and, more recently and more importantly, due to advancing technologies and decreasing sequencing costs that are allowing us to produce more data at lower cost, we have asked for a no-cost extension for the completion and broadening of the scope of the work. These supplementary investigations are expected to include the measurement of movement and behavioral patterns in PB-treated fish as indicators relevant to the pathology of Gulf War Illness. For this purpose, in lieu of a second microscope, we have requested permission to allot funds for the purchase of the ZebraBox and VideoTrack video recording equipment and analysis software from ViewPoint Sciences, Inc. With the additional 24 months that we requested in the no-cost extension, we anticipate that we will be also able to do more thorough validation of our

primary results through the use of in situ hybridization of whole embryos and slices from adult fish embedded in PaxGene for localization of gene expression patterns. Finally, we also are working to use other high-throughput methodologies, such as whole exome sequencing and reduced representation bisulfite sequencing (RRBS), to complement the RNA measurements. As costs continue to drop, whole exome sequencing may be the most efficient method for identifying the mutations from an ENU-mutagenesis screen that may result in increased resistance to PB and RRBS provides an in-depth assessment of DNA methylation patterns, which might be particularly useful in characterizing the nature of the long-term effects that result from acute exposures. Within HudsonAlpha and the Myers lab, we have previous experience performing these experiments and analyzing data from these cutting-edge methodologies.

Thus far in our work we have achieved the following: i) growth of an aquaculture facility with breeding colonies of several strains of zebrafish, ii) exposure of fish of multiple strains to multiple doses of pyridostigmine bromide with comparisons of gene expression patterns, iii) treatment of adult zebrafish with multiple doses of pyridostigmine bromide with comparisons of molecular effects in the head vs. the body, iv) initiation of a study of the effects of stress, and v) investigation of the specific effects of pyridostigmine bromide by comparison to other acetylcholinesterase inhibitors.

Growth of an aquaculture facility with breeding colonies of several strains of zebrafish.

The aquaculture facility was successfully completed in June 2010 and we have successfully maintained colonies of five strains of zebrafish, comprising three commonly used laboratory strains (TU, AB, and SJD), and the *ache*^{tm205} and *ache*^{tf222a} strains. With the exception of a small starter population of TU mating pairs, all strains were received as embryos and were grown to mating maturity. Sexual maturity in zebrafish commonly occurs as early as 3 months of age, however breeding is often most optimal between 6 and 18 months of age.

The common laboratory strains have been used in our initial study of the effects of PB with respect to natural variation in genetic backgrounds. The AB strain is also the genetic background for the *ache* mutant strains. The mutant strains contain nonsense and missense mutations in the gene for AChE; the nonsense mutation in the *ache*^{tm205} strain results in a stop codon prior to the catalytic site of AChE, whereas the missense mutation of the *ache*^{tf222a} strain causes the replacement of the highly conserved Gly198 residue near the active site with arginine. Both mutations are predicted to substantially reduce or eliminate AChE and homozygotes do not survive past 5-7 dpf (9). The ZebraBox and VideoTrack equipment would be expected to be useful in screening for homozygous mutants based on altered movement patterns early in development. We plan to use the *ache* mutant strains to further the understanding of the effects of PB that cannot be attributed to its primary mechanism of action for the protection against nerve agents, i.e. binding to the active site. Identifying these primary vs. off-target effects will be useful in determining which effects may be inherent in this method of protection against nerve agents and which effects could possibly be abrogated by use of alternative treatments with greater specificity or be targeted by a secondary therapeutic agent.

Exposure of fish of multiple strains to multiple doses of pyridostigmine bromide with comparisons of gene expression patterns.

Our first large-scale experiment has been conducted with the goal of characterizing the dose-dependent effects of PB as well as determining if different strains of zebrafish had different responses to the drug. Pooled groups of 20 larvae from each of three common laboratory strains – AB, TU, and SJD – were treated with solutions of 0, 0.001, 0.01, 0.1, 1, or 10 mmol PB/L for 72-h. The treatment period corresponded to the period of 4-7 days post-fertilization (dpf), the period immediately following completion of organogenesis and hatching of the embryo. At 7 dpf, larvae were euthanized by placement on ice and samples were prepared by washing twice with ice-cold PBS followed by the splitting of the sample for protein sample preparation using the PARIS Kit (Ambion, Inc) and isolation of RNA using Trizol and RNEasy reagents. RNA samples were prepared into libraries for mRNA-seq using the Nextera protocol (10) and sequenced as paired-end 50-bp on an Illumina HiSeq. Sequencing resulted in 9-43 million reads per sample that were alignable to the exons of the zebrafish genome (Zv9). Acetylcholinesterase (AChE) activity was measured by the Amplex Red Acetylcholinesterase Assay (Invitrogen, Inc) for determination of the efficacy of treatment and to identify the clinically relevant doses.

Pyridostigmine bromide treatment significantly inhibited AChE activity in a dose-dependent manner in all three strains of zebrafish (**Figure 1**). Of the 72 samples collected ($n=4$ for each treatment-strain combination), 36 samples have been sequenced and an additional 24 libraries have been prepared and submitted for sequencing. Data analysis is ongoing but preliminary results indicate that there are significant differences between the strains and that these differences are greater than the effects of PB. With this partial data set and threshold for the false discovery rate of 0.05, we have found 30 and 5989 changes in transcript levels attributable to the effects of PB and strain, respectively; there were no significant treatment-strain interactions. We used the DAVID Functional Annotation Tool (11, 12) to characterize the differences between strains, which identified the major terms associated with differentially expressed genes as those involved in detoxification, i.e. cytochrome P450 and oxidoreductases; DNA and ncRNA metabolic processes; and ribosome biogenesis (**Table 1**).

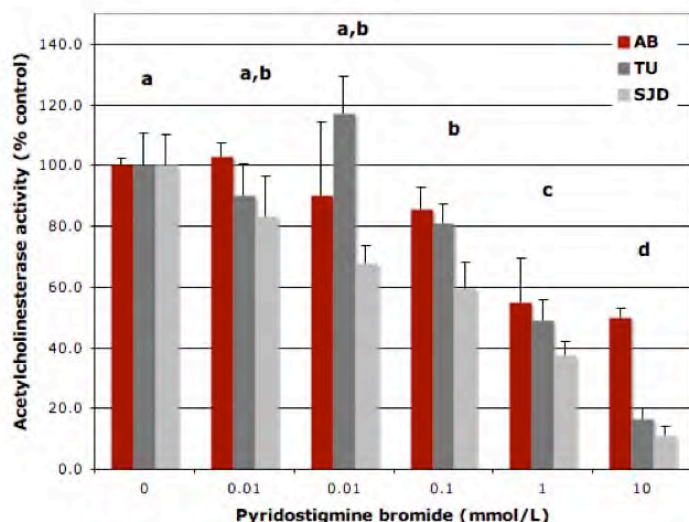


Figure 1. Acetylcholinesterase activity was inhibited by 72-h treatment of larval zebrafish with pyridostigmine bromide. Given no statistically significant effect by strain, different letters denote a statistically significant difference in acetylcholinesterase activity between doses. Data are means \pm s.e.

Strain	Direction of change	Relative to strain	Changed transcripts*	DAVID analysis terms**
SJD	downregulated	AB	1023	secondary metabolites biosynthesis, transport, and catabolism; DNA replication
	upregulated		758	-
TU	downregulated	AB	1234	DNA replication and metabolic process, microtubule, oxidoreductase, glycolysis, cell cycle, collagen
	upregulated		968	oxidoreductase, ribosome biogenesis
TU	downregulated	SJD	842	-
	upregulated		821	oxidoreductase, ncRNA metabolic process, ribosome biogenesis

Table 1. Strain-specific differences in mRNA transcripts in zebrafish.

*FDR < 0.01

**Benjamini adjusted p < 0.01

Given the multitude of differences between strains, subsequent analyses were undertaken within each strain to identify dose-dependent effects and those effects specifically associated with treatments near the clinically relevant range of ~30-50% inhibition of AChE activity (1). Within each of the three strains, a minimum of 142 genes were changed in response to PB treatment ($p < 0.05$, **Table 2**). Although few of these changes currently meet the criteria of a false discovery rate < 0.05, we are confident that with the additional statistical power contributed by the samples yet to be sequenced, many of these changes will obtain statistical significance. In addition to the functional categories found in **Table 2**, differentially expressed genes due to PB treatment also clustered into categories that are biologically plausible based on the known mechanism of action of PB, e.g. myosin, actin, and muscle contraction; calcium ion binding; and neurotransmitter transport.

Strain	Model	Dose	FDR < 0.05	p < 0.05	DAVID analysis terms*
AB	Regression	0.001, 0.01, 0.1, 1, 10 mmol PB/L	2	919	aminoacyl-tRNA synthetase, interferon regulatory factor
SJD	Regression	0.001, 0.01, 0.1, 1, 10 mmol PB/L	1	1679	protein biosynthesis, nucleotide binding
TU	Regression	0.001, 0.01, 0.1, 1, 10 mmol PB/L	0	169	-
AB	ANOVA	0 vs. 0.1 and 1 mmol PB/L	0	217	-
SJD	ANOVA	0 vs. 0.01 and 0.1 mmol PB/L	0	142	oxidoreductase
TU	ANOVA	0 vs. 0.1 and 1 mmol PB/L	0	215	-

Table 2. Analysis of differentially expressed genes within each zebrafish strain across all treatment groups and as a comparison between the control and clinically relevant doses.

*Benjamini adjusted p < 0.01

Lastly, as an additional means of preliminary evaluation of the results, we made comparisons to the work by Whistler et al. (13) in which changes in gene expression from the peripheral mononuclear blood cells of Gulf War veterans (9 with Gulf War Illness, 11 controls) were assayed by microarray. Of the 82 probe sets that were associated with the disease, 67 human genes were represented, of which 45 genes had a direct homolog in zebrafish. Based on our partial data set, we found 20 transcripts with a tendency towards a relationship with PB dose or a difference at the clinically relevant

doses in at least one strain ($p < 0.10$), suggesting that our experimental results have relevance to long-term effects observed in Gulf War Illness patients. Upon completion of sequencing, we will begin validation of the sequencing results by real-time RT-PCR. Additionally, given the epidemiological evidence that consumption of an increased number of PB pills was associated with increased risk of GWI and severity of symptoms, a subsequent study is being initiated in which larvae are exposed to the fish water or the clinically relevant dose of PB for 8, 24, 72, or 168-h.

Treatment of adult zebrafish with multiple doses of pyridostigmine bromide with comparisons of molecular effects in the head vs. the body.

Previous studies have suggested that the response to toxins is very similar in larvae and adults when using zebrafish as a model organism (14). However, to verify this result for PB, we conducted a preliminary study of the response to PB in adult zebrafish of the AB strain. Adult zebrafish (8 months of age) were removed from the communal tank and placed into individual beakers with 300 mL of fish water and allowed to acclimate for 3 days before the initiation of a 72-h treatment with solutions of 0, 0.1, 1, 10, 100, or 1000 $\mu\text{mol PB/L}$. One male and one female fish were treated at each dose. At the end of the treatment period, the fish were euthanized by submersion in ice water followed by decapitation with a razor blade. The head and the body of the fish were used for isolation of protein and RNA, measurement of AChE activity and mRNA-seq as described for larvae in the previous section. Alignment to the RefSeq exons of the zebrafish resulted in 21-48 million reads per sample for downstream analyses.

Consistent with reports that PB is unable to cross the blood-brain barrier, there were no significant effects of PB treatment on AChE activity in the head. However, AChE activity in the body was significantly inhibited in adult zebrafish treated with either 10 or 1000 $\mu\text{mol PB/L}$ relative to controls (**Figure 2**). Taken together with observations that the dose-response to AChE activity was much stronger in the male fish than the female fish ($R^2 = 0.71$ vs. 0.14), we chose to do mRNA profiling by mRNA-seq using samples from the heads and bodies of the male fish treated with 0, 10, and 1000 $\mu\text{mol PB/L}$. Using unsupervised hierarchical clustering based on the 293 most variable genes, samples clustered first by the region of the body (**Figure 3**). Within the tree node representing the body samples, the PB-treated samples clustered away from the control, whereas the differences were much smaller among the samples from the head, with no separation of the PB-treated samples. Our statistical analysis of the all expressed genes confirmed that the largest effects were due to the region from which the sample originated, with 2884 transcripts being differentially expressed between the head and the body ($\text{FDR} < 0.05$). As expected, transcripts that were significantly differentially

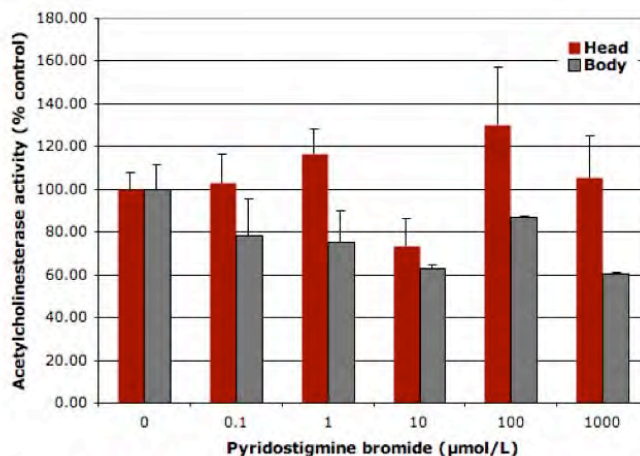


Figure 2. Acetylcholinesterase in the body of adult zebrafish was inhibited by 72-h treatment with 10 and 100 $\mu\text{mol/L}$ pyridostigmine bromide, whereas no significant differences in activity were observed in the head. Data are means \pm s.e.

expressed with at least 50% greater expression in the head were enriched for genes associated with the eyes and brain, whereas the enriched ZFIN anatomy terms associated with greater expression in the body included trunk musculature and the whole organism. Likely due to current lack of replicates, there were no transcripts that met this strict criteria ($FDR < 0.05$) for the main effect of PB treatment or for the treatment:region interaction. However, 906 mRNAs were altered by PB treatment and 1086 mRNA had a response to PB that also depended on the origin of the sample when using the more relaxed threshold of $p < 0.05$.

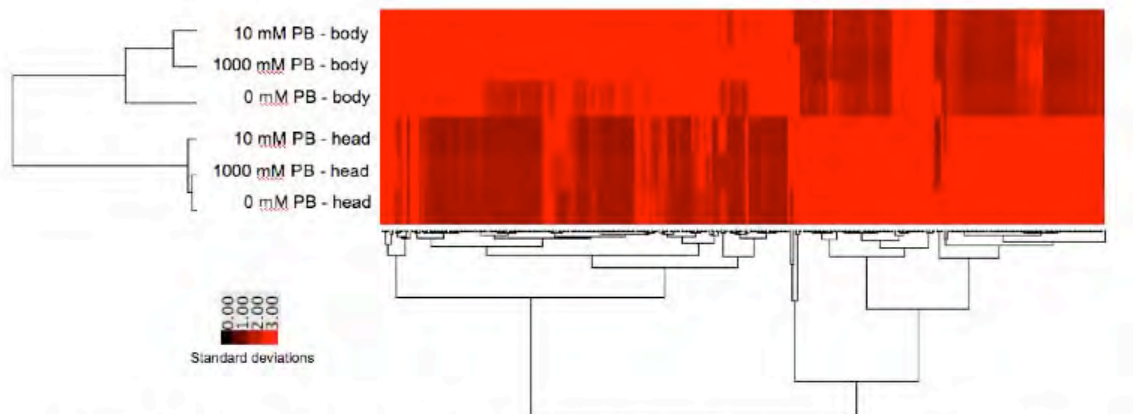


Figure 3. Clustering of the head apart from the body of adult zebrafish, with greater distances between PB-treated vs. untreated samples from the body as compared to those obtained from the head.

Investigation of the specific effects of pyridostigmine bromide by comparison to other acetylcholinesterase inhibitors.

The interaction between stress and exposure to PB remains controversial, with the most recent evidence suggesting that the effects of PB, including neurological effects, are exacerbated through some unknown peripheral mechanism rather than through increased permeability of the blood-brain barrier. To gain further insight into this phenomenon, we have proposed to study the effects of PB with and without concurrent stressors. Our studies of the dose-dependent effects of PB in both larval and adult zebrafish have provided valuable information for the design of the forthcoming study, however we needed to validate our ability to induce a stressful situation in zebrafish with a measurable outcome. Thus, we conducted a small study of the dose-response to alarm pheromone in both male and female adult zebrafish. Alarm pheromone was collected from two euthanized adult fish by the methods of Cachat et al (15). Adult TU zebrafish (12 months old) were exposed to different amounts of alarm pheromone in a total volume of 300 mL in a beaker for 30 min. Given that introduction to the beaker may also induce stress due to the novelty of the situation, one male was also taken directly from a communal tank as an experimentally naïve control. After precisely 30 min of treatment, fish were euthanized by submersion in ice water, followed by decapitation. Samples were stored at -80°C prior to the extraction and measurement of whole body cortisol, using a minimally modified extraction protocol and the High Sensitivity Human Salivary Cortisol ELISA Kit (Salimetrics, Inc) as described by Cachat et al (15). Consistent with expectations, whole body cortisol concentrations generally rose with increasing doses of alarm pheromone and the lowest cortisol concentration was observed in the experimentally naïve male (**Figure 4**). When data from just the males

was considered, the response was dose-dependent ($R^2=0.99$, $p = 0.018$). However, analysis of the data of both sexes without normalization by body weight, revealed an equally strong relationship between dose and cortisol concentrations ($R^2=0.91$, $p = 0.016$), suggesting that females produced similar absolute amounts of cortisol in response to alarm pheromone, but due to the added weight of carrying eggs, whole body cortisol concentrations tended to be lower when normalized by body weight. Ultimately, these results imply that we were able to successfully utilize these protocols to induce a stressful condition in adult zebrafish and measure the response using whole body cortisol concentrations.

The preliminary dose-response experiments for both PB and alarm pheromone have provided the data necessary for the initiation of a larger study using adult zebrafish, expected to be initiated in early 2012. Treatment groups in this study will include control, PB, stressed, and PB-stressed groups ($n = 6$ fish). Adult male zebrafish (6-12 months of age) will be exposed to solely to the treatment tank (control), 1000 mmol PB/L solution for 72-h (PB), and/or 30-min daily exposure to alarm pheromone solution (stressed). Fish will be euthanized at the end of treatment, as well as 1-, 2-weeks, and 1-month post-treatment. Four samples will be collected for the isolation of protein, RNA, and DNA and two fish will be used for the determination of whole body cortisol concentrations.

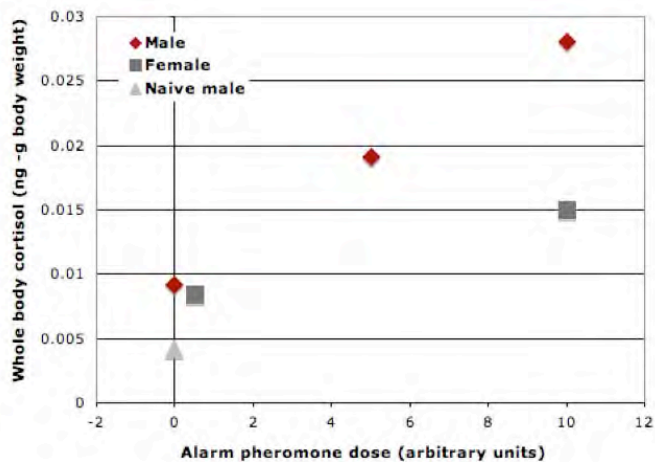


Figure 4. Whole body cortisol concentrations relative to exposure to alarm pheromone. Each data point represents one sample.

Investigation of the specific effects of pyridostigmine bromide by comparison to other acetylcholinesterase inhibitors.

We have proposed to identify off-target effects of pyridostigmine by genetic means using the *ache^{tm205}* and *ache^{tf222a}* strains of zebrafish. However, this may also be addressed using toxicological methodologies whereby we compare the gene expression profiles between larval zebrafish treated with PB and other acetylcholinesterase inhibitor (AChEI) treatments. Galantamine (GAL, brand name Razadyne), rivastigmine (RIV, brand name Exelon), and donepezil (brand name Aricept) are all water-soluble AChEIs that are currently approved by the FDA for the treatment of Alzheimer's disease and have been studied for their use against nerve agents. In addition to advancing our understanding of the specific effects of pyridostigmine bromide, it may be desirable to consider these other AChEIs as potential alternatives for pretreatment during the threat of nerve agent exposure. With this goal in mind, we conducted a comparative study of the effects of PB, GAL, and RIV in larval zebrafish. The treatment period lasted 72-h (4-7 dpf), all treatment solutions were prepared at a concentration of 0.1 mmol/L with fish water used for treatment of the control group. Each treatment had 5 biological replicates of 20 pooled TU larvae. Donepezil was also included in the study, but at 0.1 mmol/L the donepezil solution resulted in 100% mortality of larvae within 24-h after administration.

At the end of the treatment period, protein and RNA samples were prepared, AChE activity was quantified, and mRNA-seq libraries were prepared as described previously herein. The mRNA-seq libraries (n = 3 per treatment, one RIV sample currently in queue) were sequenced as single-end 36-bp on an Illumina GAllx, producing ~9-30 million reads alignable to Zv9 exons per sample.

The treatment of larval zebrafish with AChEIs was successful in inhibiting AChE activity (**Figure 5**). Acetylcholinesterase was inhibited to approximately the range of clinical

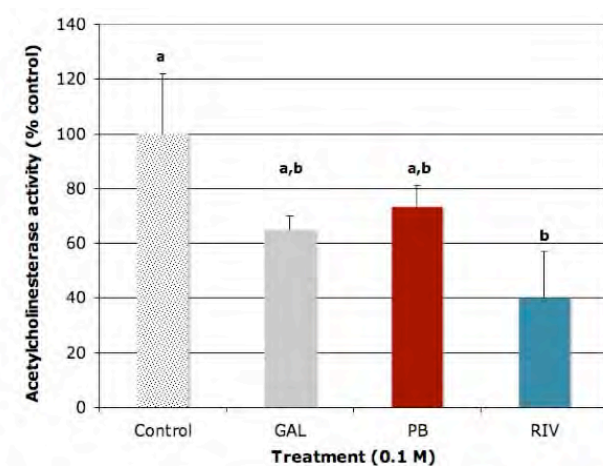


Figure 5. Acetylcholinesterase activity was inhibited by 72-h treatment of larval zebrafish with acetylcholinesterase inhibitors. Data are means \pm s.e.

relevance for GAL and PB treatments. Rivastigmine treatment produced 60% inhibition of AChE activity, which was significantly different from the control. Similar to the pattern for AChE activities, the greatest number of differentially expressed transcripts relative to control was found in response to RIV treatment, with both GAL and PB also inducing alterations in gene expression, but to a lesser extent (**Figure 6**). Not unsurprisingly, there is some degree of overlap in the genes affected by treatment with the AChEIs; however, there were many transcripts that were unique to each

drug and the analysis of these similarities and differences between therapeutic AChEIs is ongoing.

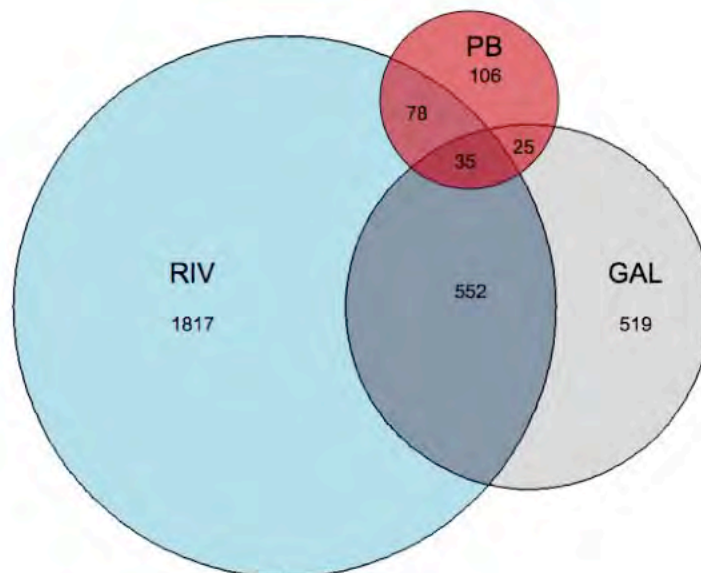


Figure 6. Treatment of larval zebrafish with acetylcholinesterase inhibitors results in differential gene expression. Numbers indicate differentially expressed transcripts ($p < 0.05$) for each substance, shaded areas of overlap indicate genes that were changed by multiple drugs.

KEY RESEARCH ACCOMPLISHMENTS

During the first part of this research program we have:

- Built an aquaculture facility with active breeding colonies of 5 strains of zebrafish.
- Characterized the response to pyridostigmine bromide (PB) biochemically by determining acetylcholinesterase activity levels in the whole larval fish and in both the heads and bodies of adult animals.
- Investigated treatment of adult zebrafish with alarm pheromone for the induction of stress and measured the response by assaying whole body cortisol concentrations.
- Conducted preliminary analyses of mRNA-seq data from larval and adult zebrafish treated with several concentrations of PB, as well as compared the response to PB vs. two other acetylcholinesterase inhibitors in larvae.
- Identified strain-specific differences in larval zebrafish gene expression between 3 commonly used laboratory strains – AB, TU, and SJD.

REPORTABLE OUTCOMES

We anticipate that we will begin presenting this data at seminars, symposia, and conferences in early 2012. Preparation and submission of a manuscript is expected by mid-2012.

CONCLUSION

In summary, we have been consistently making progress towards an increased understanding of the effects of pyridostigmine bromide and factors of relevance to the combat situation in the 1991 Gulf War, such as stress and genetic variation. We have characterized the dose-response to treatment with PB in larval and adult zebrafish biochemically by measuring changes in acetylcholinesterase activities and via transcriptional profiling with mRNA-seq. With our gene expression data sets we have identified differences in transcript levels attributable to response to PB, as well as natural variation among three strains of zebrafish, between samples taken from different regions of the fish, and from treatment with two additional acetylcholinesterase inhibitors. We anticipate being able to initiate a large study of the effect of PB and/or stress in adult zebrafish in the coming months. Subsequent goals include validation of results, characterization of motility and behavioral effects, and use of additional methodologies to more thoroughly understand how the long-term effects of Gulf War Illness resulted from transient exposures. Finally, we will begin testing additional chemical toxins for their effects on genomic measurements in zebrafish.

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APPENDICES AND SUPPORTING DATA

None.